(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
22 March 2001 (22.03.2001)

PCT

(10) International Publication Number
WO 01/20327 A1

(51) International Patent Classification[7]: G01N 33/542, 33/60, C12Q 1/48

(21) International Application Number: PCT/GB00/03495

(22) International Filing Date: 13 September 2000 (13.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
9921684.8 15 September 1999 (15.09.1999) GB

(71) Applicant *(for all designated States except MG, US)*: ASTRAZENECA AB [SE/SE]; S-151 85 Sodertalje (SE).

(71) Applicant *(for MG only)*: ASTRAZENECA UK LIMITED [GB/GB]; 15 Stanhope Gate, London W1Y 6LN (GB).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: WADDELL, Ian, David [GB/GB]; Alderley Park, Macclesfield, Cheshire SK10 4TG (GB).

(74) Agent: BRYANT, Tracey; Astrazeneca, Global Intellectual Property, Alderley Park, P.O. Box 272, Mereside, Macclesfield, Cheshire SK10 4GR (US).

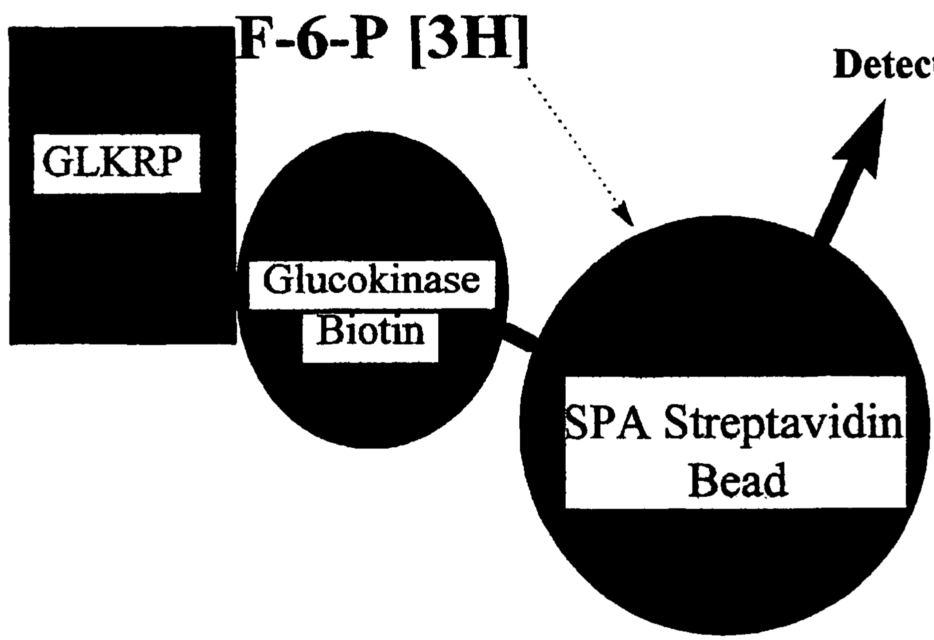
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

[Continued on next page]

(54) Title: SPA IN A SCREENING METHOD FOR MODULATORS OF GLUCOKINASE



(57) Abstract: This invention relates to assay methods which measure the binding interactions between glucokinase (GLK), the regulatory protein GLKRP, and fructose-6-phosphate. The methods are useful for identifying compounds which modulate glucokinase. Such compounds may have utility in the treatment of non-insulin dependent diabetes.

WO 01/20327 A1

— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

ASSAYS

The present invention relates to assay methods suitable for identifying compounds which stimulate glucokinase in pancreatic β-cells, leading to a decreased threshold for insulin secretion. In addition the compounds are expected to lower blood glucose by increasing hepatic glucose uptake. Such compounds may have utility in the treatment of non-insulin dependent diabetes (NIDDM).

In the pancreatic β-cell and liver parenchymal cells, the main plasma membrane glucose transporter is GLUT2. Under physiological glucose concentrations, GLUT2 is not rate limiting with the effect that in these cells the rate of glucose uptake is limited by the rate of phosphorylation of glucose to glucose-6-phosphate (G-6-P), catalysed by glucokinase (GLK) (Pilkis and Granner 1992, Malaisse 1993). GLK has a high (6-10mM) Km for glucose, is not inhibited by physiological concentrations of G-6-P and is predominantly expressed in liver, kidney and pancreatic β-cells (Pilkis *et al* 1994 and Caro et al 1995).

Recent developments have suggested that GLK may have an important role in the development of NIDDMs (Glasser et al 1998). Firstly, GLK mutations are believed to be the primary defect responsible for several forms of maturity onset diabetes of youth (MODY) a rare form of NIDDM (Froguel et al 1993, Bell et al 1996 and Shiota et al 1998). Secondly, the constitutive expression of yeast hexokinase gene in transgenic mice has been shown to cause increased insulin secretion and hypoglycaemia. This data offers new evidence in support of a critical role for GLK in determining glucose phosphorylation and insulin secretion rate in the beta cell.

GLK is associated with a regulatory protein (GLKRP) which binds to and inactivates GLK in the presence of fructose-6-phosphate (F-6-P). Fructose-1-phosphate (F-1-P) and inorganic phosphate interfere with the binding of F-6-P thereby preventing binding of GLKRP to GLK. (See Figure 1). A compound which similarly interferes with the interaction between GLK and GLKRP will effectively stimulate GLK and may offer an opportunity for the development of a novel therapeutic agent for the treatment of NIDDM (Veiga-da-Cunhe *et al* 1996).

In the hepatocyte GLK acts as the rate limiting step in glucose uptake and utilisation. A compound which stimulates GLK by interfering with the interaction between GLK and GLKRP will lower blood glucose by increasing hepatic glucose uptake. In pancreatic β cells

increased glucose metabolism leads through increased β cell [ATP]/[ADP] concentration ratio, to closure of the ATP-dependant K^+ channels, hence to membrane depolarisation and opening of voltage gated Ca^{2+} channels. The ensuing increase in β cell Ca^{2+} provokes a glucose sensitive insulin release (Matschinsky et al 1993). A compound which stimulates GLK by interfering with the interaction between GLK and GLKRP in β cells will thus also lead to a decreased glucose threshold for insulin secretion. Both effects will be of benefit in the treatment of NIDDM.

Enzymatic activity of GLK may be measured by incubating GLK, ATP and glucose as shown in Figure 2. The rate of product formation may be determined by coupling the assay to a G-6-P dehydrogenase, NADP/NADPH system and measuring the increase in optical density at 340nm (Matschinsky et al 1993).

However, this type of assay procedure has a number of disadvantages.

Firstly, use of such an assay method to screen compounds would identify all compounds which modulate enzyme activity, regardless of their mechanism of action. For example the assay would detect compounds which directly activate glucokinase in the absence of the GLKRP, compounds which prevent F-6-P binding or which act as F-1-P mimetics. In addition the spectrophotometric assay would equally well detect compounds which inhibited a protein to protein interaction between GLK and GLKRP.

Secondly, it is possible that false positives could be identified by the assay, i.e. compounds which appear to increase glucokinase activity but in reality do not. For example compounds which are capable of stimulating the coupling enzyme(s), or which have significant absorbance at 340nm would not be distinguished in this assay from compounds which act by stimulating glucokinase itself.

Thus, there is a need for new assay methods which are able to provide information on the precise mechanism of action of compounds which modulate GLK activity. In particular, assay methods are needed to identify compounds which stimulate GLK activity by preventing the binding interaction between GLK and GLKRP. Such assay methods are provided by the GLK/GLKRP binding assay and the F-6-P/GLKRP binding assay disclosed in the present invention.

In the present invention we have developed new methods for identifying compounds which modulate GLK. In particular, we have developed an assay system which identifies compounds which modulate GLK by preventing the interaction between GLK and GLKRP.

It is to be understood that whenever an assay system as described herein is referred to, a method and/or process utilising said assay system is also contemplated.

The assay system comprises two related assay methods; a GLK/GLKRP binding assay and a F-6-P / GLKRP binding assay.

The GLK/GLKRP binding assay provides an assay method for measuring the binding interactions between GLK and GLKRP. The method may be used to identify compounds which modulate GLK by modulating the interaction between GLK and GLKRP.

GLKRP and GLK are incubated with an inhibitory concentration of F-6-P, optionally in the presence of test compound, and the extent of interaction between GLK and GLKRP is measured. Compounds which either displace F-6-P or in some other way reduce the GLK/GLKRP interaction will be detected by a decrease in the amount of GLK/GLKRP complex formed. Compounds which promote F-6-P binding or in some other way enhance the GLK/GLKRP interaction will be detected by an increase in the amount of GLK/GLKRP complex formed. (See Figure 3).

The F-6-P / GLKRP binding assay provides an assay method for measuring the binding interaction between GLKRP and F-6-P. This method may be used to provide further information on the mechanism of action of the compounds.

Compounds identified in the GLK/GLKRP binding assay may modulate the interaction of GLK and GLKRP either by displacing F-6-P or by modifying the GLK/GLKRP interaction in some other way. For example, protein-protein interactions are generally known to occur by interactions through multiple binding sites. It is thus possible that a compound which modifies the interaction between GLK and GLKRP could act by binding to one or more of several different binding sites.

The F-6-P / GLKRP binding assay identifies only those compounds which modulate the interaction of GLK and GLKRP by displacing F-6-P from its binding site on GLKRP.

GLKRP is incubated with test compound and an inhibitory concentration of F-6-P, in the absence of GLK, and the extent of interaction between F-6-P and GLKRP is measured. Compounds which displace the binding of F-6-P to GLKRP may be detected by a change in the amount of GLKRP/F-6-P complex formed. (See Figures 4 and 5).

Therefore according to one aspect of the invention we provide an assay method which method comprises measurement of the binding interaction between GLKRP and either GLK or F-6-P.

In a preferred embodiment the assay method comprises:

(i) contacting

(a) GLKRP or a homologue or fragment thereof, and

(b) GLK or a homologue or fragment thereof, and / or

(c) an inhibitory concentration of F-6-P,

in the presence and absence of a test compound;

and

(ii) determining the binding interaction between (a) and one of either (b) or (c);

and

(iii) determining whether the test compound modulates the binding interaction determined in (ii).

Assay Method 1

A particularly preferred embodiment of the assay methods of the invention is a scintillation proximity assay (SPA).

SPA involves the use of fluomicrospheres coated with acceptor molecules such as enzymes or receptors, to which a ligand will bind selectively in a reversible manner (Bosworth and Towers, 1989). The technique requires the use of a ligand labelled with an isotope that emits low energy radiation which is dissipated easily into an aqueous medium. At any point during an assay, bound labelled ligands will be in close proximity to the fluomicrospheres, allowing the emitted energy to activate the fluor and produce light. In contrast, the vast majority of unbound labelled ligands will be too far from the fluomicrospheres to enable the transfer of energy. Bound ligands produce light but free ligands do not, allowing the extent of ligand binding to be measured without the need to separate bound and free ligand.

It will be appreciated that a scintillation proximity assay may be used in either or both the GLK/GLKRP binding assay and the F-6-P/GLKRP binding assay.

Therefore in a further aspect of the invention we provide a scintillation proximity assay for measuring the interaction between GLK and GLKRP, wherein one of (a), (b) or (c) as defined in *Assay Method 1* is radiolabelled and another of (a), (b) or (c) as defined in *Assay Method 1* is bound to a fluomicrosphere.

In a particularly preferred embodiment, the radiolabelled ligand is [^{3}H]F-6-P. In another particularly preferred embodiment GLK is biotinylated and the fluomicrospheres are coated with streptavidin. Biotinylated GLK binds to the streptavidin coated fluomicrospheres as shown in Figure 3. Complex formation between GLK, GLKRP and [^{3}H]F-6-P may be detected by the emission of light from the fluomicrospheres. Compounds which modulate the interaction between GLK and GLKRP may be identified by a change in light emission from the fluomicrospheres.

In a further aspect of the invention we provide a scintillation proximity for measuring the interaction between F-6-P and GLKRP, wherein one of (a) or (c) as defined in *Assay Method 1* is radiolabelled, and another of (a) or (c) as defined in *Assay Method 1* is bound to a fluomicrosphere and (b) as defined in *Assay Method 1* is omitted,

In a preferred embodiment of the scintillation proximity assay for measuring the interaction between F-6-P and GLKRP, the radiolabelled ligand is [^{3}H]F-6-P. In a particularly preferred embodiment (a) as defined in *Assay Method 1* is labelled with a FLAG tag, and the fluomicrospheres are coated with an anti-FLAG antibody.

In a further particularly preferred embodiment, (a) as defined in *Assay Method 1* is biotinylated, and the fluomicrospheres are coated with streptavidin.

Binding of F-6-P to GLKRP may be detected by the emission of light from the fluomicrospheres. Compounds which displace F-6-P from its binding site on GLKRP may be identified by a change in light emission from the fluomicrospheres.

Compounds which may be tested in the assays include simple organic molecules, commonly known as "small molecules", for example those having a molecular weight of less than 2000 Daltons. The assay may also be used to screen compound libraries such as peptide libraries, including synthetic peptide libraries and peptide phage libraries. Other suitable molecules include antibodies, nucleotide sequences and any other molecules which stimulate GLK.

Modulation of activity comprises either activation or inhibition. Thus a compound which modulates GLK is a compound which either stimulates or inhibits GLK. The terms "modulator of GLK" and "GLK modulator" are also used herein to refer to a compound that either stimulates or inhibits GLK. The compounds of the invention have utility in the treatment of NIDDMs, in general this would arise by stimulation of GLK.

An inhibiting concentration of F-6-P is one which reduces the rate of the GLK enzyme reaction. Preferably the rate should be reduced by greater than 85%. This reduction would typically be achieved using a concentration of F-6-P of 25μM (when used with rat GLKRP) and 10μM (when used with human GLKRP).

In a preferred embodiment, step (i) of *Assay Method 1* is carried out in the presence of glucose. Any convenient concentration of glucose may be used for example 1, 2, 5, 10, 50 or 100 mM. In a particularly preferred embodiment the concentration of glucose is 5 mM.

A particular advantage of the assays of the invention is that they are very convenient to use. The assays can be carried out in 96-well microplates allowing large numbers of compounds to be tested simultaneously.

Preferably, GLK and GLKRP used in the assays of the invention are human proteins.

By homologue we mean a protein with a similar amino acid sequence to a GLK protein sequence or a GLKRP protein sequence as set out in Tanizawa et al 1991 and Bonthron, D.T. *et al* 1994 respectively. The homologue may be a protein from the same species, i.e. a homologous protein family member. Alternatively, the homologue may be a similar protein from a different species such as rat or mouse, useful in providing animal models of NIDDMs. Convenient homologues include those which share a sequence similarity of 70% or greater with a GLK or GLKRP sequence set out in Tanizawa et al 1991 and Bonthron, D.T. *et al* 1994 respectively. Preferred sequence similarities include 75% and 80% identity, other preferred sequence similarities include 85% and 90% identity, further preferred sequence similarities include 95% identity.

Fragments as used herein include peptides containing six or more consecutive amino acids of the GLK and GLKRP sequences set out in (Tanizawa et al 1991 and Bonthron, D.T. *et al* 1994) respectively. Preferably the fragments possess the same or essentially the same biological activity as the full length molecules from which they are derived. The fragments may represent for example more than 1% or more than 5% or more than 10% or more than 50% or more than 90% of the full length molecules from which they are derived.

It will be appreciated that there are a number of alternative procedures which may be used to measure the GLK/GLKRP binding interaction and the F-6-P / GLKRP binding interaction in the methods of the present invention. Such procedures include rapid filtration of equilibrium binding mixtures, enzyme linked immunosorbent assays (ELISA), radioimmunoassays (RIA) and fluorescence resonance energy transfer assays (FRET). For

further information on FRET the reader is directed to International Patent Application WO 94/28166 (Zeneca). Methods to identify potential drug candidates have been reviewed by Bevan *et al*, 1995.

Once a compound is identified then medicinal chemistry techniques may be applied to further refine its properties, for example to enhance efficacy and/or reduce side effects. Therefore in a further aspect of the invention we provide a compound which modulates the interaction between (a) and one of either (b) or (c) in *Assay Method 1*.

In a further aspect of the invention we provide a novel modulator of GLK, or a pharmaceutically acceptable salt thereof, for use in a method of treatment of NIDDM of the human or animal body by therapy.

In a further aspect of the invention we provide a pharmaceutical composition which comprises a novel modulator of GLK, or a pharmaceutically acceptable salt thereof, in association with a pharmaceutically-acceptable diluent or carrier.

The composition may be in a form suitable for oral use, for example a tablet, capsule, aqueous or oily solution, suspension or emulsion; for topical use, for example a cream, ointment, gel or aqueous or oily solution or suspension; for nasal use, for example a snuff, nasal spray or nasal drops; for vaginal or rectal use, for example a suppository; for administration by inhalation, for example as a finely divided powder such as a dry powder, a microcrystalline form or a liquid aerosol; for sub-lingual or buccal use, for example a tablet or capsule; or for parenteral use (including intravenous, subcutaneous, intramuscular, intravascular or infusion), for example a sterile aqueous or oily solution or suspension. In general, the above compositions may be prepared in a conventional manner using conventional excipients.

The invention also includes a method of treating NIDDM or a medical condition mediated alone or in part by GLK which comprises administering to a warm-blooded animal requiring such treatment an effective amount of a GLK modulator as defined above.

The invention also provides the use of a GLK modulator in the production of a medicament for use in the treatment of NIDDM.

The size of the dose of a GLK modulator, for therapeutic or prophylactic purposes will naturally vary according to the nature and severity of the NIDDM, the age and sex of the patient and the route of administration, according to well-known principles of medicine.

In using a novel GLK modulator for therapeutic or prophylactic purposes it will generally be administered so that a daily dose in the range for example 0.5mg to 75mg per kg body weight is received, given if required in individual doses. In general lower doses will be administered when a parenteral route is employed. Thus, for intravenous administration, a dose in the range for example 0.5mg to 30mg per kg body weight will generally be used. Similarly, for administration by inhalation, a dose in the range for example 0.5mg to 25mg per kg body weight will be used.

The invention will now be illustrated but not limited by reference to the following Examples and Figures.

Figure 1

Figure 1 shows the uptake of glucose into a cell by the plasma membrane glucose transporter GLUT2, and its conversion to glucose-6-phosphate (G-6-P) by glucokinase (GLK). Enzyme activity is down-regulated by the glucokinase regulatory protein (GLKRP).

The binding interaction between GLK and GLKRP is enhanced by fructose-6-phosphate (F-6-P) resulting in decreased glucokinase activity and reduced glucose metabolism.

Fructose-6-phosphate is displaced from its binding site on GLKRP by fructose-1-phosphate (F-1-P), leading to dissociation of GLK and GLKRP and resulting in increased glucokinase activity and stimulation of glucose metabolism.

Figure 2

Figure 2 shows a reaction pathway in which glucose is phosphorylated to G-6-P by glucokinase in the presence of ATP. By coupling the reaction to G-6-P dehydrogenase, the rate of the reaction may be determined by measuring the reduction of NADP to NADPH, detected as a change in optical density at 340nm.

Figure 3

Figure 3 shows a preferred embodiment of a GLK/GLKRP binding assay. The diagram illustrates the emission of light from streptavidin coated fluomicrospheres on formation of a binding complex between biotinylated GLK, GLKRP and [^{3}H]F-6-P.

Figure 4

Figure 4 shows a preferred embodiment of a F-6-P / GLKRP binding assay. The diagram illustrates the emission of light from fluomicrospheres coated with protein A and an anti-FLAG antibody, on formation of a binding complex between FLAG-tagged GLKRP and [^{3}H]F-6-P.

Figure 5

Figure 5 is a titration curve showing the displacement of F-1-P by F-6-P, obtained using the GLK/GLKRP scintillation proximity assay of the invention.

REFERENCES

Bell et al (1996) *Ann Rev Physiol* **58** 171-186

Bevan P et al (1995) *TIBTECH* **13** 115

Bonthron, D.T. et al (1994) Hum Mol Genet **3** 1627-1631

Bosworth N and Towers P *Nature* **341** 167-168 1989

Caro et al (1995) *Horm Metab Reg* **27** 19-22

Freund (1956) *Adv Tuberc Res* 7 130-148

Froguel et al (1993) New England J Med **328** 697-702

Glaser et al (1998) *New England J Med* **338** 226-230

Harlow E. and Lane D. (1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Chapter 5, pp92-114

Malaisse W.J. (1993) *Endocrinologia* **40** 309-313

Matschinsky et al (1993) *J Clin Invest* **92** 2092-2096

Pilkis and Granner (1992) *Ann Rev Physiol* **54** 885-909

Pilkis et al (1994) *J Biol Chem* **269** 21925-21925

Sambrook J, Fritsch EF & Maniatis T, (1989), *Molecular Cloning, A Laboratory Manual,* Cold Spring Harbor Laboratory Press

Shiota M et al (1998) *Diabetes* **47** 867-873

Short, J.M. et al (1998) *Nucleic Acids Research* **16** 7583-7590

Tanizawa, Y. et al (1991) *PNAS USA* **88** 7294-7297

Veiga-da-Cunha et al (1996) *Diabetologia* **39** 1173-1179

Yanisch-Perron C et al (1985) *Gene* **33** 109-119

EXAMPLES

Example 1

Production of recombinant GLK and GLKRP

Preparation of mRNA

Human liver total mRNA was prepared by polytron homogenisation in 4M guanidine isothiocyanate, 2.5mM citrate, 0.5% Sarkosyl, 100mM β-mercaptoethanol, followed by centrifugation through 5.7M CsCl, 25mM sodium acetate at 135,000g (max) as described in Sambrook J, Fritsch EF & Maniatis T, 1989.

Poly A^+ mRNA was prepared directly using a FastTrack™ mRNA isolation kit (Invitrogen).

PCR amplification of GLK and GLKRP cDNA sequences

Human GLK and GLKRP cDNA was obtained by PCR from human hepatic mRNA using established techniques described in Sambrook, Fritsch & Maniatis, 1989. PCR primers were designed according to the GLK and GLKRP cDNA sequences shown in Tanizawa et al 1991 and Bonthron, D.T. *et al* 1994.

Cloning in Bluescript II vectors

GLK and GLKRP cDNA was cloned in E. coli using pBluescript II, (Short et al 1998) a recombinant cloning vector system similar to that employed by Yanisch-Perron C *et al* (1985), comprising a colEI-based replicon bearing a polylinker DNA fragment containing multiple unique restriction sites, flanked by bacteriophage T3 and T7 promoter sequences; a filamentous phage origin of replication and an ampicillin drug resistance marker gene.

Transformations

E coli transformations were generally carried out by electroporation. 400 ml cultures of strains DH5α or BL21(DE3) were grown in L-broth to an OD 600 of 0.5 and harvested by centrifugation at 2,000g. The cells were washed twice in ice-cold deionised water, resuspended in 1ml 10% glycerol and stored in aliquots at -70°C. Ligation mixes were desalted using Millipore V series™ membranes (0.0025mm) pore size). 40μl of cells were

incubated with 1μl of ligation mix or plasmid DNA on ice for 10 minutes in 0.2cm electroporation cuvettes, and then pulsed using a Gene Pulser™ apparatus (BioRad) at 0.5kVcm^{-1}, 250μF, 250 ½. Transformants were selected on L-agar supplemented with tetracyline at 10μg/ml or ampicillin at 100μg/ml.

Expression

GLK was expressed from the vector pTB375NBSE in E.coli BL21 cells, producing a recombinant protein containing a 6-His tag immediately adjacent to the N-terminal methionine. Alternatively, another suitable vector is pET21(+)DNA, Novagen, Cat number 697703. The 6-His tag was used to allow purification of the recombinant protein on a column packed with nickel-nitrilotriacetic acid agarose purchased from Qiagen (cat no 30250).

GLKRP was expressed from the vector pFLAG CTC (IBI Kodak) in E.coli BL21 cells, producing a recombinant protein containing a C-terminal FLAG tag. The protein was purified initially by DEAE Sepharose ion exchange followed by utilisation of the FLAG tag for final purification on an M2 anti-FLAG immunoaffinity column purchased from Sigma-Aldrich (cat no. A1205).

Example 2

Biotinylation of GLK

GLK was biotinylated by reaction with biotinamidocaproate N-hydroxysuccinimide ester (biotin-NHS) purchased from Sigma-Aldrich (cat no. B2643). Briefly, free amino groups of the target protein (GLK) are reacted with biotin-NHS at a defined molar ratio forming stable amide bonds resulting in a product containing covalently bound biotin. Excess, non-conjugated biotin-NHS is removed from the product by dialysis. Specifically, 7.5mg of GLK was added to 0.31mg of biotin-NHS in 4mL of 25mM HEPES pH7.3, 0.15M KCl, 1mM dithiothreitol, 1mM EDTA, 1mM $MgCl_2$ (buffer A). This reaction mixture was dialysed against 100mL of buffer A containing a further 22mg of biotin-NHS. After 4hours excess biotin-NHS was removed by extensive dialysis against buffer A.

Example 3

GLK/GLKRP scintillation proximity assay

Recombinant human GLK and GLKRP were used to develop a "mix and measure" 96 well SPA (scintillation proximity assay). (A schematic representation of the assay is given in Figure 3). GLK (Biotinylated) and GLKRP are incubated with streptavidin linked SPA beads (Amersham) in the presence of an inhibitory concentration of radiolabelled [3H]F-6-P (Amersham Custom Synthesis TRQ8689), giving a signal as depicted in Figure 3. Compounds which either displace the F-6-P or in some other way disrupt the GLK / GLKRP binding interaction will cause this signal to be lost.

Binding assays were performed at room temperature for 2 hours. The reaction mixtures contained 50mM Tris-HCl (pH 7.5), 2mM ATP, 5mM $MgCl_2$, 0.5mM DTT, recombinant biotinylated GLK (0.1 μg), recombinant GLKRP (0.1 μg), 0.05mCi [3H] F-6-P (Amersham) to give a final volume of 100μl. Following incubation, the extent of GLK/GLKRP complex formation was determined by addition of 0.1mg/well avidin linked SPA beads (Amersham) and scintillation counting on a Packard TopCount NXT.

Example 4

F-6-P / GLKRP scintillation proximity assay

Recombinant human GLKRP was used to develop a "mix and measure" 96 well scintillation proximity assay. (A schematic representation of the assay is given in Figure 4). FLAG-tagged GLKRP is incubated with protein A coated SPA beads (Amersham) and an anti-FLAG antibody in the presence of an inhibitory concentration of radiolabelled [3H]F-6-P. A signal is generated as depicted in Figure 4. Compounds which displace the F-6-P will cause this signal to be lost. A combination of this assay and the GLK/GLKRP binding assay will allow the observer to identify compounds which disrupt the GLK/GLKRP binding interaction by displacing F-6-P.

Binding assays were performed at room temperature for 2 hours. The reaction mixtures contained 50mM Tris-HCl (pH 7.5), 2mM ATP, 5mM $MgCl_2$, 0.5mM DTT, recombinant FLAG tagged GLKRP (0.1 μg), Anti-Flag M2 Antibody (0.2μg) (IBI Kodak), 0.05mCi [3H] F-6-P (Amersham) to give a final volume of 100μl. Following incubation, the extent of F-6-P/GLKRP complex formation was determined by addition of 0.1mg/well

protein A linked SPA beads (Amersham) and scintillation counting on a Packard TopCount NXT.

Example 5

Production of human anti-GLKRP antibodies

Purified recombinant human FLAG-tagged GLKRP protein was used in conjunction with Freund's adjuvant (Freund, 1956) to produce antibodies in New Zealand white rabbits. Methods for producing antibodies are well known in the art and are described in Harlow and Lane 1988.

The GLKRP protein antibodies or fragments thereof can be used to decrease the inappropriately enhanced inhibition of GLK by GLKRP which occurs in certain pathological conditions including diabetes.

CLAIMS

1. An assay method which comprises measurement of the binding interaction between GLKRP and either GLK or F-6-P.

2. An assay method according to claim 1 which method comprises:
   (i) contacting
      (a) GLKRP or a homologue or fragment thereof, and
      (b) GLK or a homologue or fragment thereof, and / or
      (c) an inhibitory concentration of F-6-P,
      in the presence and absence of a test compound;
   and
   (ii) determining the binding interaction between (a) and one of either (b) or (c);
   and
   (iii) determining whether the test compound modulates the binding interaction determined in (ii).

3. A method as claimed in claim 2 wherein one of either (a), (b) or (c) is radiolabelled and another of (a), (b) or (c) is bound to a fluomicrosphere.

4. A method as claimed in claim 3 wherein (c) is [^{3}H]F-6-P.

5. A method as claimed in claim 3 or claim 4 wherein (b) is biotinylated and the fluomicrospheres are coated with streptavidin.

6. A method as claimed in any one of claims 2 to 5 wherein (b) is omitted.

7. A method as claimed in claim 3 wherein (a) is labelled with a FLAG tag, (b) is omitted, and the fluomicrospheres are coated with an anti-FLAG antibody.

8. A method as claimed in claim 3 wherein (a) is biotinylated, (b) is omitted, and the fluomicrospheres are coated with streptavidin.

9. A method as claimed in any one of claims 2 to 8 in which step (i) is carried out in the presence of glucose.

10. A compound which modulates the interaction between (a) and one of either (b) or (c) in the method of claim 2.

11. Use of a compound as claimed in claim 10 in the production of a medicament for use in the treatment of NIDDM.

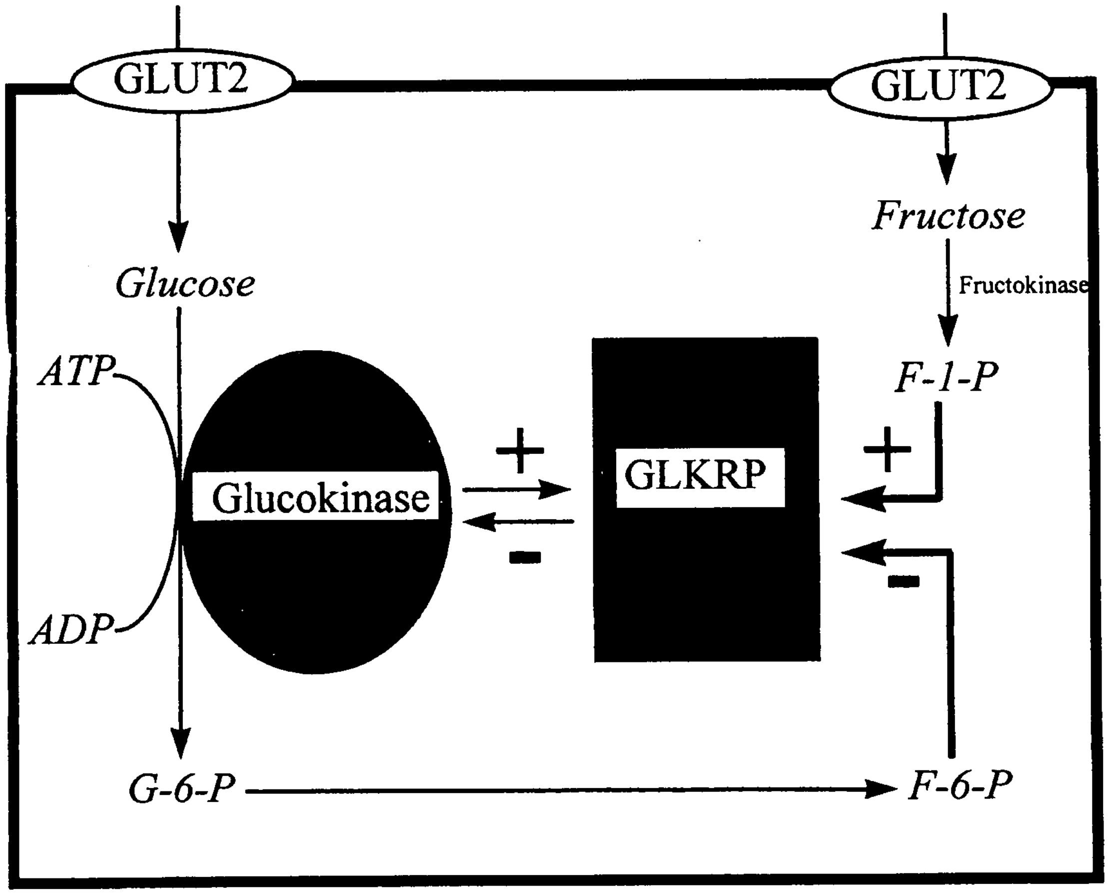

GLUT2
GLUT2
Fructose
Fructokinase
Glucose
F-1-P
ATP
+
+
Glucokinase
GLKRP
-
-
ADP
G-6-P
F-6-P

FIGURE 1

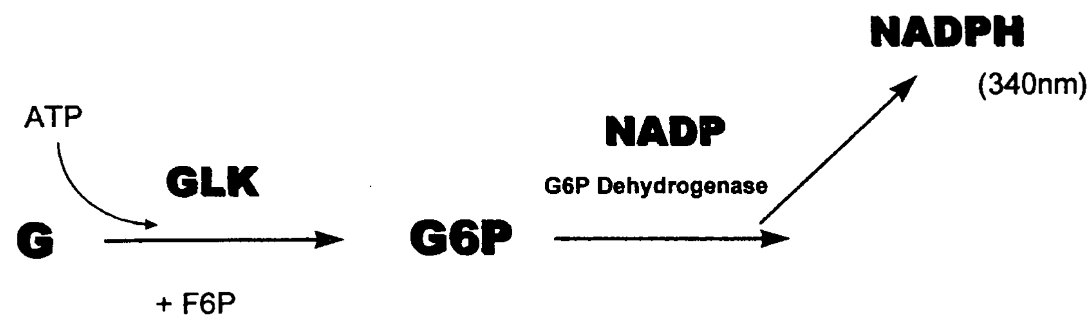


FIGURE 2

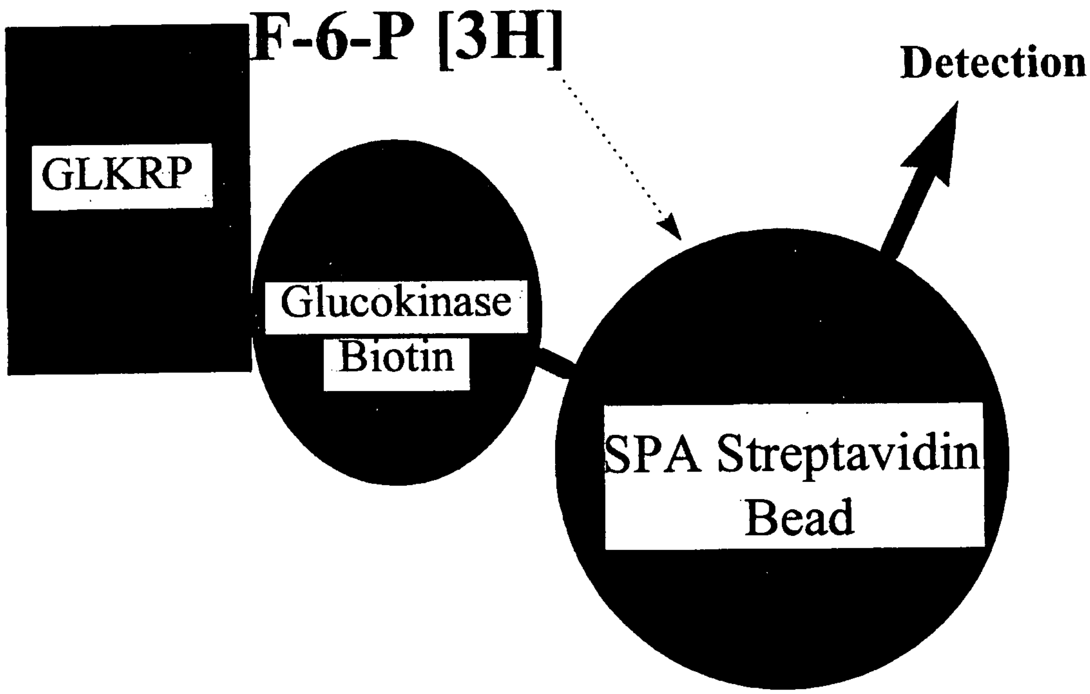

F-6-P [3H]
Detection
GLKRP
Glucokinase
Biotin
SPA Streptavidin
Bead

FIGURE 3

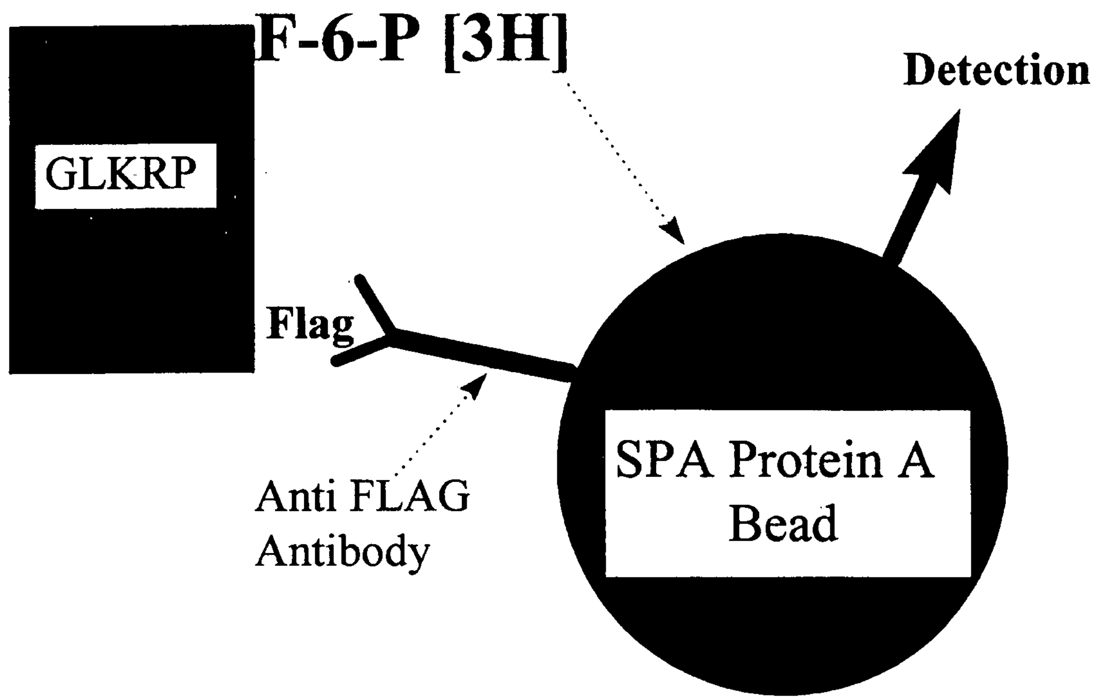

F-6-P [3H]
Detection
GLKRP
Flag
Anti FLAG
Antibody
SPA Protein A
Bead

FIGURE 4

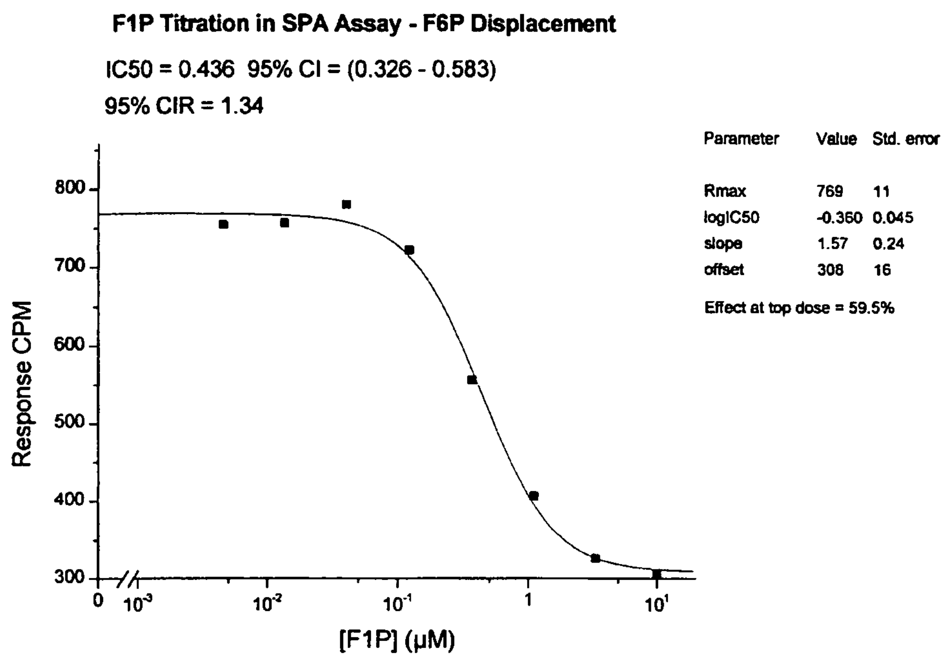

F1P Titration in SPA Assay - F6P Displacement
IC50 = 0.436 95% CI = (0.326 - 0.583)
95% CIR = 1.34
Parameter Value Std. error
Rmax 769 11
logIC50 -0.360 0.045
slope 1.57 0.24
offset 308 16
Effect at top dose = 59.5%
Response CPM
800
700
600
500
400
300
0
10^{-3}
10^{-2}
10^{-1}
1
10^{1}
[F1P] (μM)

FIGURE 5

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/03495

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/542 G01N33/60 C12Q1/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, PAJ, WPI Data, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VAN SCHAFTINGEN, E ETAL.: "A protein from rat liver confers to glucokinase the property of being antagonistically regulated by fructose-6-phosphate and fructose-1-phosphate" EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 179, no. 1, 15 January 1989 (1989-01-15), pages 179-184, XP000980294 abstract page 180, left-hand column, line 27 -right-hand column, line 21 page 182, left-hand column, line 25 -page 183, right-hand column, line 16; figures 5-7 --- -/--	1,2,9,10

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 January 2001	13/02/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Tuynman, A

5

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VANDERCAMMEN A ET AL: "THE MECHANISM BY WHICH RAT LIVER GLUCOKINASE IS INHIBITED BY THE REGULATORY PROTEIN" EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 191, no. 2, 1990, pages 483-490, XP000980287 ISSN: 0014-2956 abstract	1,2,9,10
X	DETHEUX M ET AL: "EFFECTORS OF THE REGULATORY PROTEIN ACTING ON LIVER GLUCOKINASE A KINETIC INVESTIGATION" EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 200, no. 2, 1991, pages 553-562, XP000980285 ISSN: 0014-2956 abstract page 556; figure 4 page 558; table 2	1,2,10
X	VAN SCHAFTINGEN EMILE ET AL: "Short-term control of glucokinase activity: Role of a regulatory protein." FASEB JOURNAL, vol. 8, no. 6, 1994, pages 414-419, XP002158048 ISSN: 0892-6638 abstract page 416, left-hand column, line 37 -right-hand column, line 7 page 418, right-hand column, line 26 - line 48	1,2,9-11
A	WO 93 21330 A (ZYMOGENETICS INC) 28 October 1993 (1993-10-28) page 24, line 35 -page 25, line 2	3,4
A	WO 98 20024 A (MERCK FROSST CANADA INC ;FRIESEN RICHARD (CA); ZAMBONI ROBERT (CA)) 14 May 1998 (1998-05-14) figure 1 page 27, line 22 - line 25	5-8

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 10,11 relate to compounds or the use of these compounds defined by reference to a desirable characteristic or property, namely that these compounds are modulators of the interaction between glucokinase and glucokinase regulatory protein.
The claims cover all compounds having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only one such compound. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compound by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the compound mentioned in the description, page 1, line 24-26; page 2, line 16; page 8 lines 18-20; page 9, lines 8-9; Figure 1 and Figure 5, being fructose-1-phosphate.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Ir. .ational Application No
PCT/GB 00/03495

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9321330 A	28-10-1993	NONE	
WO 9820024 A	14-05-1998	EP 0941232 A	15-09-1999
		US 6066715 A	23-05-2000